



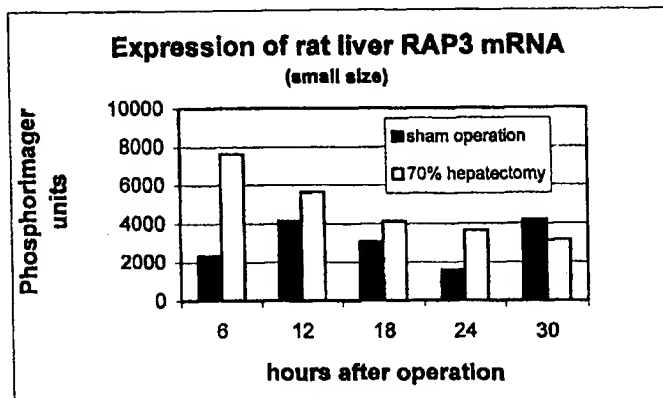
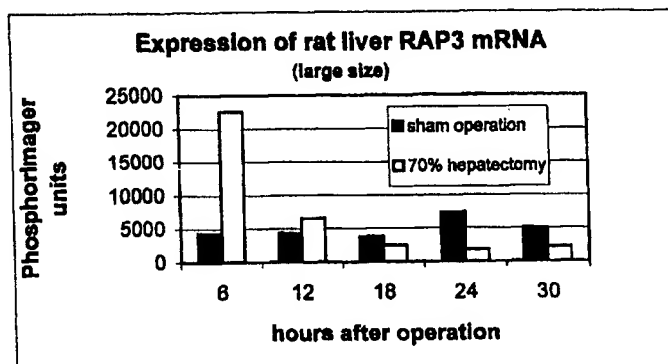
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(54) Title: GENE AND PROTEIN INVOLVED IN LIVER REGENERATION

## (57) Abstract

The present invention relates to a gene involved in regeneration processes of the liver and comprising a nucleotide sequence which is at least 70 % homologous to the sequence of figure 1 or the sequence of figure 6, or the complementary strand thereof, for use in the design of PCR probes for detecting nucleotide sequences in a source material, which nucleotide sequences represent genes corresponding with the gene sequence of figure 1 or the sequence of figure 6; protein encoded by said gene for use in diagnosis of liver regeneration and/or liver cell proliferation; and antibodies directed against this protein, a PCR primer comprising at least part of said gene as a probe, a single stranded nucleotide sequence being at least in part complementary to the messenger RNA transcribed from said gene as a probe an expression vector and a host cell comprising said nucleotide sequence, for use in a method for detecting the occurrence of liver cell proliferation in a subject.



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## GENE AND PROTEIN INVOLVED IN LIVER REGENERATION

The present invention relates to the detection of a novel gene and protein involved in liver cell proliferation. The gene and protein and related molecules, such as nucleotide probes derived from the gene and antibodies directed to the protein form also part of the invention. The gene will be identified herein as RAP3 gene. The corresponding protein is called RAP3 protein.

The adult liver has the capacity to regenerate after damage or partial resection. This process may allow for recovery from hepatic injuries caused by viruses, toxins, ischemia, surgery, and auxiliary liver transplantation. Liver regeneration has been studied extensively in the rat after a 70% partial hepatectomy. During the first four hours following partial hepatectomy there is a rapid, transient transcriptional activation of genes involved in the immediate early response. After induction of these immediate early genes during the transition from the quiescent state of the liver ( $G_0$ ) to the growth phase ( $G_1$ ), a delayed early gene activation is initiated which peaks during the transition of the  $G_1$  to the DNA synthesis phase (S phase).

In the research that led to the present invention novel genes involved in the delayed early response were identified by analyzing gene expression in rat liver at six hours after 70% partial hepatectomy. Upregulated genes were selected by cDNA subtractive hybridization. Upregulation was quantified by Northern blotting and the truly upregulated genes were characterized by sequence analysis.

Twelve genes were found to be upregulated at different degrees (1.5 to 10.4 fold) six hours after partial hepatectomy. Sequence analysis revealed that eight of the upregulated genes have previously been reported to be associated with liver regeneration or cell proliferation in general, one has previously been assigned an unrelated function and three have no sequence similarity to known genes.

The various upregulated genes showed two distinct gene expression patterns during a 30 hour period after partial hepatectomy. The first pattern has two peaks coincident with the G<sub>1</sub> phases of two consecutive hepatic cell cycles. The second one shows a narrow peak at six hours after which the gene is downregulated. The novel RAP3 gene (RAP: regeneration associated protein) which was most upregulated (3.3 fold), showed the latter gene expression pattern.

10           The full length cDNA of this gene was isolated from a rat liver cDNA library. Sequence analysis showed two full length cDNAs of 1282 and 1834 bp, respectively, encoding a novel protein of 367 amino acid residues.

Figures 1A and 1B show the nucleotide sequence of the 15 cDNA's. Figure 2 shows the derived amino acid sequence.

In addition, the cDNA of the human RAP3 gene was isolated. Sequence analysis showed two cDNAs of 1282 and 1867 bp respectively, encoding a protein of 363 amino acids. Figures 6A and 6B show the nucleotide sequence of 20 the cDNA's. Figure 7 shows the derived amino acid sequence. The 1867 human RAP3 cDNA shows a 76% homology with the 1834 rat RAP3 cDNA.

On the basis of this finding it became possible to design probes, primers and reagents for use in 25 diagnosis. Probes and primers are generally

based on the nucleotide sequence of the genes.

Hybridization probes can comprise the whole or a large part of the coding or complementary strand of the sequence. PCR primers are typically smaller and encompass 30 about between 10 and 50, preferably between 15 and 30, more preferably about 20 nucleotides.

The nucleotide sequences of some suitable PCR primers are given in the following table.

35 Table I

primer name	nucleotide sequence
F1RAP	5' GCA TCG TGG AAA GCA TGG CT 3'
F215RAP	5' GGG ACC CTT GAG AGA GCC TG 3'

F371RAP	5' CTT GAG GCA GCA GTT GAA AC 3'
F571RAP	5' TCC ACC CTT ATG CAG AAC GC 3'
F771RAP	5' AGT ACC TTC ATC CGT GTC AG 3'
F971RAP	5' CGC CTT CGC TCC AGA GTT GG 3'
5 F1171RAP	5' AGG GTG GAG GGT CCT GCA TA 3'
F1371RAP	5' GCA AGC CAG TAC TTG ACC GT 3'
F1621RAP	5' GTG GTC CTG CTG GGG GAT CA 3'
R234RAP	5' CAG GCT CTC TCA AGG GTC CC 3'
R420RAP	5' CTA CCT GCT CCA TCA GCT CG 3'
10 R570RAP	5' AGA GTT CTT TGA CTC GGT CC 3'
R770RAP	5' GAG CTC ATC TCG CAG CTG AT 3'
R970RAP	5' CTG TGG CTA GGC GGG GGT GG 3'
R1170RAP	5' CTG CCT ATT AGG CCA TGC TG 3'
R1370RAP	5' AGT CAG TCT CCC CCG CAC AC 3'
15 R1570RAP	5' TGG CAG GGA TGT ACA CAC TC 3'
R1837RAP	5' TTT CCA TCA TGA GCG TCT AT 3'

The hybridization probes can be labeled with a detectable label, such as a radioactive or biotin label.

20           Diagnosis of expression of the gene can be performed by means of a Northern blot. Total RNA or mRNA of a sample is separated on an agarose gel. The separation pattern is transferred to a nylon or nitrocellulose filter. An increase or decrease in the  
25 expression level is subsequently detected by hybridization with the above described hybridization probe. Typically a reference sample is included for comparison.

In case the protein is the basic macromolecule  
30 for diagnosis polyclonal or monoclonal antibodies are used for detection. The skilled person is very well capable of preparing such antibodies based on his common knowledge. Antibodies against the protein are part of the present invention.

35           Samples to be diagnosed can be a liver biopsy, plasma or serum. The latter can be used because the protein is secreted in the blood stream.

With the above described diagnostic methods an increase or decrease in the expression of the gene of the invention can be detected. The information that can thus be obtained is useful for establishing the efficacy of  
5 therapeutic agents stimulating liver regeneration and for patients who underwent an (auxiliary) liver transplantation and for monitoring patients treated with a bioartificial liver.

The invention is further illustrated in the  
10 following examples, which are in no way intended to be limiting to the invention. In the examples reference is made to the following figures:

Figure 1A is the nucleotide sequence of the  
1282 bp cDNA.

15 Figure 1B is the nucleotide sequence of the  
1834 bp cDNA.

Figure 2 shows the deduced amino acid sequence  
of the rap3 protein.

Figure 3 shows a polyacrylamide gel of liver  
20 cDNA fragments before and after subtraction. 26 cDNA  
fragments were found to be enriched after subtraction.  
Some of these are indicated by arrows. Lane 1 shows liver  
cDNA fragments of 6 hours 70% partial hepatectomy before  
subtraction. Lane 2 shows cDNA fragments of 6 hours 70%  
25 partial hepatectomy after subtraction.

Figure 4 shows the results of the Northern blot  
analysis of the temporal expression of RAP3 up to 30  
hours after 70% partial hepatectomy. Panel A represents  
the Northern blot mRNA expression patterns at 3, 6, 12,  
30 18, 24 and 30 hours after the 70% hepatectomy (hpx) and  
laparotomy (sham). Panel B represents the quantified  
hybridization signals indicated in PhosphorImager  
arbitrary units obtained at 6, 12, 18, 24 and 30 hours  
after the 70% hepatectomy and laparotomy.

35 The novel gene RAP3 is mostly upregulated 6  
hours after partial hepatectomy after which it becomes  
downregulated.

Figure 5 shows a rat tissue Northern blot hybridized with a RAP3 cDNA probe. The RAP3 gene is specifically expressed in the liver.

Figure 6A shows the nucleotide sequence of the 5 1282 bp human RAP3 cDNA.

Figure 6B shows the nucleotide sequence of the 1867 bp human RAP3 cDNA (B).

Figure 7 shows the deduced amino acid sequence of the human rap3 protein.

10 Figure 8 shows the results of immunoblotting. About 6 ng of denatured rat RAP-3 protein was blotted on each strip. The strips were incubated with two antisera in various concentrations: 1-3: antiserum against purified native rat RAP3 protein (concentrations: 1:2000, 15 1:8000, 1:32000); 4-7: antiserum against purified denatured rat RAP3 protein (1:2000, 1:8000, 1:32000, 1:128000).

Figure 9 shows the results of an immunoblot after analysis with the program LumiAnalyst. 2.5  $\mu$ l rat 20 plasma was blotted per lane. The blot was incubated with antiserum against the denatured RAP3 protein. (hpx: plasma after 70% partial hepatectomy; sham: plasma after sham operation; positive control: about 10 ng purified denatured rat RAP3 protein).

25

## **EXAMPLES**

### **EXAMPLE 1**

#### **Isolation of RAP3 gene associated with liver regeneration**

##### 30 **1. Introduction**

Recovery from Hepatic injuries caused by viruses, toxins, ischemia, surgery and auxiliary liver transplantation can be achieved by regeneration of the liver. The regeneration process has been studied 35 extensively in the rat after a 70% partial hepatectomy.

During the first four hours following partial hepatectomy there is a rapid, transient transcriptional response. After this induction during the transition from

the quiescent state of the liver ( $G_0$ ) to the growth phase ( $G_1$ ), a delayed early gene activation is initiated, which peaks during the transition of the  $G_1$  to the DNA synthesis phase (S phase).

- 5           This example demonstrates the isolation and identification of genes which are upregulated in the regenerating liver 6 hours after 70% partial hepatectomy.

## 2. Methods

### 10 2.1 Rat liver tissue preparation

- Experiments were carried out in compliance with the guidelines on the care and use of laboratory animals of the University of Amsterdam. Regenerating liver was obtained from male Wistar rats (200-225 g). Rats were  
15 anesthetized with ether and subjected to midventral laparotomy. Subsequently, the left lateral and the median liver lobes were removed (70% partial hepatectomy) (G.M. Higgins and R.M. Anderson, Arch. Pathol. 12, 186 (1931)). For sham-operated animals, the liver was exposed  
20 by a midventral laparotomy.

The rats were allowed to recover from anesthesia. At 3, 6, 12, 18, 24, and 30 hours, respectively, after the 70% partial hepatectomy and sham surgery the animals were killed and the remaining liver  
25 was immediately harvested.



## 2.2 RNA isolation

Total liver RNA was isolated from liver tissue using the Trizol reagent kit (Life Technologies). Liver poly A<sup>+</sup> RNA was isolated from total liver RNA using oligo-  
5 (dT)-cellulose (Boehringer Mannheim GmbH) affinity chromatography as described previously (Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular cloning: A laboratory Manual, Cold Spring Harbor, NY). To obtain  
10 highly pure poly A<sup>+</sup> RNA populations the oligo-(dT)-cellulose step was performed twice. The integrity of the poly A<sup>+</sup> RNA populations was determined on Northern blot by hybridization with glutathione-S transferase (data not shown).

## 15 2.3 PCR-select cDNA subtraction

The PCR-select cDNA subtraction kit (Clontech) was used to selectively amplify delayed early genes differentially expressed during liver regeneration. This method subtracts sequences common to both cDNA  
20 populations by suppressing undesirable PCR amplification, rather than by physically separating single stranded and double-stranded DNA. The 6 hours 70% partial hepatectomy liver poly A<sup>+</sup> population, containing the differentially expressed mRNA's, was compared with the 6 hours  
25 laparotomy liver mRNA population. Delayed-early genes start to appear 3 to 4 hours after the 70% partial hepatectomy. By using a laparotomy liver mRNA population rather than a normal liver mRNA population, the two populations were equalized for acute phase mRNA's, which  
30 are induced by the operation itself.

The PCR-select cDNA subtraction was performed according to the manufacturer's protocol with the following modifications. After two hybridizations, a nested PCR was used to selectively amplify the  
35 differentially expressed sequences. The second, nested PCR was performed in the presence of 0.5  $\mu$ M [ $\alpha$ -<sup>33</sup>P]dATP (1200 Ci/mmol, final volume 25  $\mu$ l). Subsequently, the amplified and differentially expressed cDNA fragments

were visualized on a denaturing 4% polyacrylamide DNA sequencing gel. An X-ray film (Biomax, Kodak) was exposed overnight to the unfixed, dried gel.

Figure 3 shows the results of the subtraction. Before subtraction (lane 1), the majority of the cDNA's were poorly identifiable, indicating the presence of many cDNA fragments of different molecular size. After subtraction (lane 2), 26 distinct cDNA fragments were observed as bands that were not apparent before subtraction.

#### 2.4 Isolation and identification of visualized cDNA fragments

The 26 cDNA fragments that became visible after PCR-select cDNA subtraction were excised from the dried polyacrylamide gel and heated to 100°C for 5 minutes. Subsequently, 25 µl of the aqueous cDNA extract was used to amplify the cDNA by PCR with the nested primers used in the PCR-select cDNA subtraction. The PCR product was ligated into pCR II (Invitrogen), transformed into INVαF' competent cells, and plated out on agar plates containing ampicillin and X-Gal. Of each cloned PCR product, 6 white colonies were analyzed by PCR with T7 and SP6 primers for the presence of an insert.

Subsequently, plasmids containing an insert were purified using QIAprep (Qiagen) and the sequences of the inserts were determined using a dye terminator cycle sequencing system (Perkin Elmer) and a 377 DNA sequencer (ABI PRISM).

30

#### 2.5 Northern blot analysis

To determine whether the expression of the genes found by the PCR-select subtractive hybridization is truly increased 6 hours after partial hepatectomy, Northern blot analysis was carried out using the purified cDNA fragments as probes. Poly A<sup>+</sup> RNA samples (0.8 µg) of the liver 6 hours after the hepatectomy and sham operation were electrophoresed on a 0.22 M formaldehyde-

1% agarose gel, and blotted onto a Hybond-N nylon membrane (Amersham) by capillary transfer overnight. For fixation of the poly A<sup>+</sup> RNA the blots were baked in an oven at 80°C for 2 hours.

5           The inserts of the sequenced clones were amplified by PCR using the nested primers of the PCR-select cDNA subtraction method. Qiaquick-spin columns (Qiagen) were used to purify the PCR products. The purified PCR products were radioactively labelled  
10 according to the hexamer-random primed method following the manufacturer's protocol (Promega), purified on Qiaquick-spin columns (Qiagen), and hybridized with the blots. Prehybridization (2 hours, 42°C) and hybridization (overnight, 42°C) was performed in 5 x SSPE, 50%  
15 formamide, 5 x Denhardt, 0.5% SDS, and 0.1 mg/ml sheared heat-denatured herring sperm DNA.

Following hybridization the blots were washed with 2 x SSC and 0.1% SDS for 15 min at room temperature and 42°C, respectively. Subsequently, the solution was  
20 replaced with 1 x SSC and 0.1% SDS and the blots were washed for 15 min at room temperature and at 42°C, respectively. The amount of hybridization was analyzed and quantified using a PhosphorImager (Molecular Dynamics).

25           The fold induction of the mRNA levels observed in the 70% partially hepatectomized animals over the sham operated animals after the specific hybridization was adjusted for variability in RNA loading.

The genes which were upregulated 1.5 times or  
30 more 6 hours after 70% hepatectomy together with their identity are given in Table II. Beside these twelve genes, three genes are indicated which expression could not be detected on Northern blot. The expression of the novel RAP3 gene was found to be upregulated 3.3 fold.

Table II

GENES UPREGULATED 6 HOURS AFTER A 70%		
Identity of gene	Function	Fold
5 Fibronectin	Liver regeneration	1.8
An intracisternal-A	Liver regeneration	1.8
$\gamma$ -Actin	Liver regeneration	7
Ribophorin I	Liver regeneration	5.5, 1.7 & 2.3
10 $\alpha_2$ -Macroglobulin	Hepatocyte proliferation <u>in vitro</u>	5.4
Ribosomal Protein S5	Cell cycle	3.7 & 1.9
Ribosomal Protein L13	Cell cycle	2
Amyloid A Protein	Growth factor	10.4
15 Entactin		N.D.*
TCP-1-Containing Chaperonin related gene		1.5
31 kDa Putative		N.D.*
20 Serine/ Threonine protein kinase		
Novel RAP1**	Unknown	1.5
Novel RAP2**	Unknown	1.6
Novel RAP3**	Unknown	3.3
25 Novel RAP4**	Unknown	N.D.*

\* N.D. = not detectable on Northern blot

\*\* RAP1-3: Regeneration Associated Protein1-3)

## 30 EXAMPLE 2

### Isolation and characterization of the full length RAP3 cDNA

#### Library screening and sequence analysis

A rat liver cDNA library was prepared from poly  
35 A<sup>+</sup> RNA isolated from the rat liver 6 hours after 70%  
hepatectomy. To obtain full length cDNA, the Great  
Lengths cDNA Synthesis Kit (Clontech) was used following  
the manufacturer's protocol. The adaptor ligated full

length cDNA inserts were cloned into the mammalian expression vector pCI at the EcoRI restriction site.

After transformation into DH10B electrocompetent cells (Gibco), the cDNA library was  
5 plated at a density of about 3,000 plaques per 150-mm-diameter petri dish. Colonies were lifted onto a Hybond-N nylon membrane (Amersham). The lift was hybridized with the <sup>32</sup>P-labeled RAP3 PCR fragment prepared according to the hexamer-random primed method following the  
10 manufacturer's protocol (Promega).

Following hybridization, the lift was washed and analyzed using a PhosphorImager (Molecular Dynamics). From the nine positive clones, the plasmid DNA was purified and the sequences of the inserts were determined  
15 using a Big dye terminator cycle sequencing system (Perkin Elmer) and an ABI PRISM 377 DNA sequencer (Perkin Elmer). The RAP3 cDNA was obtained by comparing the nine sequences with the sequence of the RAP3 PCR fragment. Two possible clones were detected and the start and end of  
20 the cDNA were confirmed by 5'- and 3'-RACE reactions carried out following the protocol of the Marathon cDNA Amplification kit (Clontech).

Based on the nucleotide sequence of the clones, PCR reactions were carried out with cDNA prepared from  
25 poly A<sup>+</sup> RNA of the rat liver 6 hours after 70% hepatectomy. The PCR products comprised the whole RAP3 cDNA, of which the nucleotide sequence was determined by bidirectionally sequencing the PCR products using 20 bp primers based on the already known nucleotide sequence  
30 data of the RAP3 cDNA.

Two RAP3 cDNA molecules were detected of 1282 and 1834 bp respectively. The latter showed the same nucleotide sequence as the first, but contained an additional 552 bp nucleotide part at the 3' side.

35 The nucleotide sequence of the 1282 bp RAP3 cDNA is as shown in Figure 1A.

The nucleotide sequence of the 1834 bp RAP3 cDNA is shown in Figure 1B.

Using GCG DNA software the nucleotide sequences were translated into the amino acid sequence. By analyzing the six reading frames, the largest possible protein was chosen as the RAP3 protein. Its amino acid sequence, starting with a methionine residue and ending at a stop codon, was the most likely one to form a protein in comparison with the other smaller possible proteins. Both RAP3 cDNA molecules encode the same RAP3 protein.

10           The amino acid sequence of RAP3 protein as deduced from the nucleotide sequence is shown in Figure 2.

### 15 **EXAMPLE 3**

#### Temporal expression between 3 and 30 hours after 70% partial hepatectomy

To define the temporal expression of the RAP3 gene, mRNA levels at 3, 6, 12, 18, 24, and 30 hours after the 70% partial hepatectomy and laparotomy were analyzed by the Northern blot procedure as described in example 1. Total RNA samples (20 µg) of the rat liver isolated at the various time points were electrophoresed. The Northern blot was hybridized with a radioactively labeled probe comprising basepairs 370 to 1834 of the large RAP3 cDNA. The result of the Northern blot and the quantified expression pattern are given in Figure 4. The expression pattern is presented as the hybridization signal in PhosphorImager arbitrary units obtained at 3, 6, 12, 18, 24, and 30 hours after the 70% partial hepatectomy and laparotomy.

Both RAP3 mRNA sizes are mostly upregulated 6 hours after partial hepatectomy after which they become downregulated.

35           The same procedure was carried out with probes of the other upregulated genes obtained by the PCR-select subtraction. Two distinct gene expression patterns during the 30 hour period after partial hepatectomy were found.

The first pattern has two peaks coincident with the G<sub>1</sub> phases of two consecutive hepatic cycles. The second one shows a narrow peak at six hours after which the gene is downregulated, just like the expression pattern of the novel RAP3 gene.

#### Determination of tissue specific expression

A Northern blot was prepared to determine expression of RAP3 mRNA in different tissues. The various tissues (skeletal muscle, spleen, liver, kidney, heart, lung and brain) were isolated from a female Wistar rat (175 g). The experiment was carried out in compliance with the guidelines on the care and use of laboratory animals of the University of Amsterdam. Total liver RNA was isolated from the tissues using the Trizol reagent kit (Life Technologies). A Northern blot was prepared from 20 µg total RNA samples and Northern blot analysis was carried out as described in example 1. A radioactively labeled probe comprising basepairs 370 to 1834 of the large RAP3 cDNA was used for the hybridization. The resulting Northern blot is given in Figure 5.

The RAP3 mRNA appeared to be clearly expressed in the liver and not at any detectable level in the other examined tissues. Because of this liver specificity and the 3.3 fold upregulation six hours after hepatectomy, the novel gene RAP3 was considered to be important in the process of liver regeneration.

30

#### **EXAMPLE 4**

##### Isolation and characterization of the full length human RAP3 cDNA

The Superscript Human liver cDNA library was purchased from Life Technologies. It contained liver cDNA from a 9-year old caucasian female directionally cloned into the pCMV<sup>1</sup>SPORT vector, and transformed into DH12S cells.

The cDNA library was plated at a density of about 25,000 plaques per 150-mm-diameter petri dish. Colonies were lifted onto a Hybond-N nylon membrane (Amersham). The lift was hybridized with a <sup>32</sup>P-labeled RAP3 cDNA fragment, consisting of the overlapping nucleotide sequence of the two rat RAP3 cDNA molecules. The labeling was carried out according to the hexamer random primed method following the manufacturer's protocol (Promega). Following hybridization, the membrane was washed with low stringency and analyzed using a PhosphorImager (Molecular Dynamics).

Eight positive colonies and its near surroundings were cut from the agar plate and each transferred into 1 ml LB medium. 20 µl was plated on new agar plates and the hybridization protocol was repeated. Duplo clones of the eight positive colonies were isolated from the plate. The plasmid DNA was purified and the sequences of the inserts were determined using a Big Dye terminator cycle sequencing system (Perkin Elmer) and an ABI PRISM 377 DNA sequencer (Perkin Elmer). For sequencing initially the T7 and SP6 promotor primers were used and later on primers identical to twenty nucleotides of the sequenced part of the inserts.

By bidirectionally sequencing two human RAP3 cDNA nucleotide sequences were detected, both being presented by four of the eight examined clones. The two RAP3 cDNA molecules were 1322 and 1867 bp respectively. The latter showed the same nucleotide sequence as the first, but contained an additional 545 bp nucleotide part at the 3' side. The 1867 human RAP3 cDNA shows a 76% identity with the 1834 bp rat RAP3 cDNA.

The nucleotide sequence of the 1282 bp human RAP3 cDNA is shown in Figure 6A. The nucleotide sequence of the 1867 bp human RAP3 cDNA is shown in Figure 6B.

Using GCG DNA software the nucleotide sequences were translated into the amino acid sequence. By analyzing the six reading frames, the largest possible protein was chosen as the human RAP3 protein. Its amino



acid sequence, starting with a methionine residue and ending at a stop codon, was the most likely one to form a protein in comparison with the other smaller possible proteins. Both human rRAP3 cDNA molecules encode the same rap3 protein. The human RAP3 protein shows a 73% identity with the rat RAP3 protein, indicating that they indeed are the same protein but only expressed in different species.

The 363 residues counting amino acid sequence of the human RAP3 protein as deduced from the nucleotide sequence is shown in Figure 7.

#### EXAMPLE 5

##### Production of polyclonal antibodies against both rat and human RAP3

##### Expression of RAP3 protein

Both the rat and human cDNA sequences encoding the RAP3 protein without its presequence (the first 20 amino acid residues of the protein) were cloned into the pET-15b expression vector (Novagen) at the NdeI restriction site. The inserts were flanked by NdeI restriction sites, which had been introduced by PCR. By cloning at the NdeI site, a His-tag is expressed in front of the RAP3 protein, which is necessary for purification of the protein.

The vectors containing the RAP3 insert were transformed into the bacterial strain BL21(DE3) (Novagen). One colony was inoculated in 200 ml ampicillin containing LB medium. The medium was incubated at 37 °C. After reaching an OD<sub>600</sub> of 0.6/0.7 the medium was incubated for another four hours after which the cells were isolated.

The rat RAP3 protein was purified under non-denaturing conditions from the soluble fraction of the cells and under denaturing conditions from the insoluble fraction (inclusion bodies) of the cells. The human RAP3

protein was only purified under denaturing conditions from the insoluble fraction. The purification was carried out using His•Bind resin and columns (Novagen) following the manufacturer's protocol.

5 For identification the purified proteins were sequenced. Therefore the His-tag was removed from the proteins by cleavage with biotinylated thrombin (Novagen) following the manufacturer's protocol. The proteins were run on a 10% SDS-PAGE gel in Laemmli running buffer. The  
10 gel was blotted onto a PVDF membrane in CAPS buffer. The blot was shortly stained with Fast Blue (Pharmacia). The proteins were sequenced from blot by the Protein Research Facility Amsterdam of the E.C. Slater Institute in Amsterdam. The first thirteen amino acid residues of the  
15 expressed proteins after the His-tag were identified to be the same as the amino acid residues 21-33 of the rat and human RAP3 proteins as depicted in Figure 2 and 7 respectively. This indicates that the expected RAP3 proteins were expressed and can be used as antigens to  
20 raise polyclonal antibodies against the RAP3 proteins.

#### Immunisation

Rabbits were immunised with the various RAP3 proteins in order to obtain polyclonal antibodies against  
25 the rat and human RAP3 protein. About 200 µg purified RAP3 protein (including the His-tag) was suspended in Freund Complete Adjuvant and injected intracutaneously into 15 weeks old New Zealand White rabbits. After 2 months a booster of about 200 µg protein in Freund  
30 Incomplete Adjuvant was given intramuscular. One month later a booster of about 200 µg protein in PBS was given intramuscular and one to two weeks hereafter serum was collected from the rabbits.

The serum was tested in various concentrations for its  
35 ability to recognize the purified RAP3 protein. The sera against rat RAP3 were tested on the purified denatured rat RAP3 protein by immunoblotting. About 45 ng protein was run on a 10% SDS-PAGE gel in Laemmli running buffer.

The gel was blotted onto a PVDF membrane in Laemmli running buffer containing 20% methanol. The blot was cut into seven strips each loaded with approximately 6 ng rat RAP3 protein. The strips were blocked using Protifar (Nutricia), washed and incubated with antibodies (1 h, 20 °C) according standard methods. The strips were incubated first with the sera against the rat RAP3 protein in various concentrations ranging from 1:2,000 to 1:128,000. Secondly the strips were incubated with alkaline phosphatase labeled goat anti-rabbit immunoglobulins (DAKO). The strips were now incubated with precipitating BM purple AP substrate (Boehringer Mannheim) following the manufacturer's protocol. Reaction of alkaline phosphatase with the substrate leaves a dark purple band. The results are given in Figure 8. As shown a clear band at the right protein size appears even at the lowest concentrations. Serum from the rabbits before immunisations did not react with the RAP3 protein (data not shown). So polyclonal antibodies were obtained against the rat RAP3 protein. In the same way polyclonal antibodies against human RAP3 will be obtained.

#### EXAMPLE 6

##### Detection of RAP3 protein in rat plasma

##### Rat plasma isolation

Experiments were carried out in compliance with the guidelines on the care and use of laboratory animals of the University of Amsterdam. Regenerating liver was obtained from male Wistar rats (200-225 g). Rats were anesthetized with Hypnorm and subjected to midventral laparotomy. Subsequently, the left lateral and the median liver lobes were removed (70% partial hepatectomy) {G.M. Higgins and R.M. Anderson, Arch. Pathol. 12, 186 (1931)}. For sham-operated animals, the liver was exposed by a midventral laparotomy. The rats were allowed to recover from anesthesia. At 3, 6, 12 hours, respectively, after

the 70% partial hepatectomy and sham surgery the blood of the animals was heparinized and collected. The plasma of the rats was obtained by 5 minutes centrifugation at 1650g.

5

#### Immunoblotting

A Western blot was prepared as described before containing 2.5  $\mu$ l plasma samples. Plasma samples of 3, 6 and 12 hours after both a sham operation and a 70% partial hepatectomy were examined. The blot was blocked (using Protifar), washed and incubated with antibodies (1 h, 20 °C) according standard methods. The blot was incubated first with serum against the denatured rat RAP3 protein in a 1:6,000 dilution. Secondly the strips were incubated with horse radish peroxidase labeled goat anti-rabbit immunoglobulins (DAKO). The blot was now incubated with Lumi-light<sup>plus</sup> Western blotting Substrate (Boehringer Mannheim) following the manufacturer's protocol. Analysis with the program LumiAnalyst gave the result as shown in Figure 9. RAP3 was found to be present in the plasma, so the liver excretes RAP3 into the blood. The appearing RAP3 has a somewhat smaller molecular weight than the positive control, because the positive control contains a His-tag in front of the protein. Especially the plasma samples of 6 and 12 hours after the hepatectomy showed an increase in RAP3 protein contents compared to the sham operation. So the concentration of the RAP3 protein is elevated in rat blood after 6 to 12 hours after a 70% hepatectomy.

30

#### **EXAMPLE 7**

##### Detection of changes of the amount of the RAP3 protein in the blood circulation

In order to detect changes in the amount of the RAP3 protein in the blood circulation a specific enzyme-linked immunosorbent assay (ELISA) is developed. Specific polyclonal and/or monoclonal antibodies are raised

35

against the whole protein or a part of the protein. The protein, human or rat, is expressed in a prokaryotic or eukaryotic expression system or part of the protein is synthesized chemically. Monoclonal and polyclonal  
5 antibodies, raised in rabbits, are isolated by common techniques as described previously (Coligan, J.E., Kruisbeek, A.M., Margulies, D.M., Shevach, E.M., and Strober, W. (1994) Current Protocols in Immunology, John Wiley & Sons, Inc. Chicester, New York).

## CLAIMS

1. Gene involved in regeneration processes of the liver and comprising a nucleotide sequence which is at least 70% homologous to the sequence shown in Fig. 1 or at least 70% homologous to the sequence shown Fig. 6, or the complementary strand thereof.
2. Gene as claimed in claim 1, characterized in that its cDNA has a nucleotide sequence which is at least 70% homologous to the nucleotide sequence as depicted in Fig. 1 or at least 70% homologous to the nucleotide sequence as depicted in Fig. 6, or the complementary strand thereof.
3. Gene as claimed in claims 1 or 2 for use in the design of PCR probes for detecting nucleotide sequences in a source material, which nucleotide sequences represent genes corresponding with the gene sequence shown in Fig. 1 or with the gene sequence shown in Fig. 6.
4. Gene as claimed in claims 1 or 2 for use as a marker of liver proliferation.
5. Protein encoded by a gene as defined in claims 1 and 2 and comprising an amino acid sequence which is at least 70% homologous to the amino acid sequence given in Fig. 2 or at least 70% homologous to the amino acid sequence given in Fig. 7.
6. Protein as claimed in claim 5 having the amino acid sequence as depicted in Fig. 2 or Fig. 7.
7. Protein as claimed in claims 5 and 6 for use in diagnosis of liver regeneration and/or liver cell proliferation.
8. Antibodies directed against a protein as claimed in claims 5 and 6.
9. Antibodies as claimed in claim 7 for use in a method for detecting the occurrence of liver cell proliferation in a subject.
10. Antibodies as claimed in claim 8 or 9 which antibodies are monoclonal antibodies.

11. Antibodies as claimed in claim 8 or 9 which antibodies are polyclonal antibodies.

12. PCR primer, comprising at least part of the gene as claimed in claim 1.

13. PCR primer, comprising at least part of the nucleotide sequence as shown in Fig. 1 or at least part of the nucleotide sequence as shown in Fig. 6, or its complementary strand.

14. PCR primer as claimed in claims 12 and 13, wherein the "at least part of the nucleotide sequence" encompasses 10 to 50, preferably 15 to 30, more preferably about 20 nucleotides.

15. PCR primer as claimed in claims 12 to 14 having the nucleotide sequence as depicted in Table I or the complementary strand thereof.

16. PCR primer as claimed in claims 12 to 15 for use as a probe in a method for detecting the occurrence of liver proliferation in a subject.

17. PCR primer as claimed in claims 12 to 15 for use in the detection of gene homologous to the gene as claimed in claims 1 to 3.

18. Single stranded nucleotide sequence being at least in part complementary to the messenger RNA transcribed from a gene as claimed in claims 1 to 3.

19. Single stranded nucleotide sequence as claimed in claim 18 which is antisense RNA.

20. Single stranded nucleotide sequence being at least in part complementary to the DNA or the cDNA from a gene as claimed in claims 1 to 3.

21. Single stranded nucleotide sequence as claimed in claims 18-20, further provided with a detectable label.

22. Nucleotide sequence as claimed in claims 18 to 21 for use as a probe in a method for detecting the occurrence of liver proliferation in a subject.

23. Nucleotide sequence as claimed in claim 22, **characterized in that** the method in which the nucleotide sequence is used as a probe comprises the steps of:

a) obtaining a sample of a tissue or body fluid; and

b) detecting the amount of messenger RNA transcribed from a gene as claimed in claims 1 to 3 in that sample in comparison to a reference sample by means of the probe.

24. Nucleotide sequence as claimed in claim 23, wherein the sample is a liver biopsy, plasma or serum.

25. Nucleotide sequence as claimed in claim 18, 20 or 21 for use as a probe for screening a liver cDNA or genomic library.

26. Nucleotide vector comprising the nucleotide sequence as claimed in claims 18 to 25.

27. Expression vector comprising the nucleotide sequence as claimed in claims 18 to 25 in operative association with a regulatory element that controls expression of the nucleotide sequence in a host cell.

28. Host cell comprising the nucleotide sequence as claimed in claim 18-25.

29. Host cell comprising the nucleotide sequence as claimed in claim 18-25 in operative association with a regulatory element that controls expression of the nucleotide sequence in that host cell.

30. Transgenic animal in which the gene as claimed in claim 1 or 2 is expressed transgene comprised in the genome of the animal.

31. Transgenic animal in which expression of the gene as claimed in claim 1 or 2 is prevented or repressed.

32. Method for treating liver disorder, comprising administering a compound that modulates the expression of the gene as claimed in claim 1 or 2, or the activity of the gene product of that gene to a patient in need of such treatment.

33. Method as claimed in claim 32 wherein the compound is an antisense or ribozyme molecule that blocks translation of the target gene.



34. Method as claimed in claim 32 wherein the compound is complementary to the 5' region of the target gene and blocks transcription via triple helix formation.

35. Method as claimed in claim 32 wherein the compound is an antibody that neutralizes the activity of the target gene product.

36. Method as claimed in claim 32 wherein the compound enhances the expression of the target gene, or the synthesis or activity of the gene product.

37. Method for treating liver disorder comprising administering nucleic acid encoding the gene as claimed in claim 1 or 2 to a patient in need of such treatment.

38. Method for treating liver disorder comprising administering an effective amount of the gene product of the gene as claimed in claim 1 or 2 to a patient in need of such treatment.

39. Method for enhancing the growth or regeneration of liver tissue comprising treating the liver tissue with an effective amount of the gene product of the gene as claimed in claim 1 or 2.

40. Method as claimed in claim 39 wherein the liver tissue is extracorporeal.

41. Method as claimed in claim 39 wherein the liver tissue is intracorporeal.

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```
1  GCATCGTGGA  AAGCATGGCT  GCCGTCAATCA  CCTGGGCACT  CGCCCTCCTC
51  TCAGTGTTTG  CAACTGTACA  GCGAGGAAG  AGCTTCTGGG  AGTACTTCGG
101 CCAGAACAGC  CAGGGCAAAG  GCATGATGGG  CCAGCAGCAG  AAGCTGGCAC
151 AGGAGAGCCT  GAAAGGTAGC  TTGAGCAAG  ACCTCTACAA  TATGAACAAAT
201 TTCCTAGAAA  AGCTGGGACC  CTTGAGAGAG  CCTGGGAAGG  AGCCTCCTCG
251 GCTGGCACAG  GATCCAGAAG  GCATTGCGAA  GCAGTTGCAG  CAAGAGCTGG
301 AGGAAGTGAG  CACACGCCCTG  GAGCCCTACA  TGGCTGCAAA  GCACCAGCAG
351 GTCGGCTGGA  ACCTGGAGGG  CTTGAGGCAG  CAGTTGAAAC  CCTACACGGT
401 CGAGCTGATG  GAGCAGGTAG  GCCTGAGCGT  GCAGGATCTG  CAAGAACAGC
451 TCGCGCATGGT  GGGAAAAGGC  ACCAAGGCC  AGCTCCTGGG  GGGCGTGGAT
501 GAGGCGATGA  GCCTGCTGCA  GGATATGCAA  AGTCGAGTGC  TGCACCATAC
551 GGACCGAGTC  AAAGAACTCT  TCCACCCCTTA  TGCAGAACGC  TTGGTGA CTG
601 GAATTGGGCA  CCATGTGCAG  GAGCTGCACC  GGAGTGTTC  TCCTCACGCA
651 GTTGCCAGCC  CCGCGAGACT  CAGTCGCTGC  GTGCAGACCC  TGTCCCACAA
701 ACTCACACGT  AAGCGAAGG  ACTTGACAC  CAGCATCCAA  CGCAACCTGG
751 ATCAGCTGCG  AGATGAGCTC  AGTACCTTCA  TCCGTGTCAG  CACAGACGGG
801 GCAGACAACA  GAGACTCCCT  GGACCCCTCAA  GCTCTCTCTG  ACGAGGTCCG
```

FIG. 1A-1

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851 CCAGAGACTC CAGGCTTTTC GACATGACAC CTACCTGCAG ATCGCTGCAT  
901 TCACTCAGGC CATTGACCAG GAGACCGAGG AAATCCAGCA CCAGCTGGCA  
951 CCACCCCCGC CTAGCCACAG CGCCTTCGCT CCAGAGTTGG GACACTCAGA  
1001 CAGTAATAAG GCCCTGAGCA GACTGCAGAG CCGGCTGGAC GACCTCTGGG  
1051 AAGATATTGC CTATGGCCTT CATGACCAGG GCCATAGTCA GAATAACCCCT  
1101 GAGGGTCACT CAGGTTAACT CTGCAGCTCG TTGTCTGGAC CCTGAGCCCTT  
1151 CAGCATGGCC TAATAGGCAG AGGGTGGAGG GTCCTGCATA CTATTGGCGA  
1201 GGCCACCAAA GGTGCTGCTG CCCC AACCTG TCTGGCCTCC TCAACTCCCC  
1251 CACTCAGGTG CATTACACTC AGTAGGTTTG GC

FIG. 1A-2

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1 GCATCGTGGA AAGCATGGCT GCCGTCAATCA CCTGGGCACT CGCCCTCCTC
51 TCAGTGTTTG CAACTGTACA GCGAGGAAG AGCTTCTGG AGTACTTCGG
101 CCAGAACAGC CAGGGCAAAG GCATGATGG CCAGCAGCAG AAGCTGGCAC
151 AGGAGAGCCT GAAAGGTAGC TTGGAGCAAG ACCTCTACAA TATGAACAAT
201 TTCCTAGAAA AGCTGGGACC CTTGAGAGAG CCTGGGAAG AGCCTCCTCG
251 GCTGGCACAG GATCCAGAAG GCATTCCGAA GCAGTTGCAG CAAGAGCTGG
301 AGGAAGTGAG CACACGCCCTG GAGCCCTACA TGGCTGCAAA GCACCAGCAG
351 GTCGGCTGGA ACCTGGAGGG CTTGAGGCAG CAGTTGAAAC CTTACACGGT
401 CGAGCTGATG GAGCAGGTAG GCCTGAGCGT GCAGGATCTG CAAGAACAGC
451 TCGGCATGGT GGGAAAAGGC ACCAAGGCC AGCTCCTGGG GGGCGTGGAT
501 GAGCGGATGA GCCTGCTGCA GGATATGCAA AGTCGAGTGC TGCACCATAC
551 GGACCGAGTC AAAGAACTCT TCCACCCTTA TGCAGAACGC TTGGTGACTG
601 GAATTGGGCA CCATGTGCAG GAGCTGCACC GGAGTGTTC TCCTCACGCA
651 GTTGCCAGCC CCGCGAGACT CAGTCGCTGC GTGCAGACCC GTGCCCACAA
701 ACTCACACGT AAGCGAAGG ACTTGCACAC CAGCATCCAA CGCAACCTGG
751 ATCAGCTGCG AGATGAGCTC AGTACCTTCA TCCGTGTGAG CACAGACGGG
801 GCAGACAACA GAGACTCCCT GGACCCTCAA GCTCTCTCTG ACGAGTCCG
```

FIG. 1B-1

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851 CCAGAGACTC CAGGCTTTTC GACATGACAC CTACCTGCAG ATCGCTGCAT  
901 TCACTCAGGC CATTGACCAG GAGACCGAGG AAATCCAGCA CCAGCTGGCA  
951 CCACCCCCGC CTAGCCACAG CGCCTTCGCT CCAGAGTTGG GACACTCAGA  
1001 CAGTAATAAG GCCCTGAGCA GACTGCAGAG CCGGCTGGAC GACCTCTGGG  
1051 AAGATATTGC CTATGGCCTT CATGACCAGG GCCATAGTCA GAATAACCCT  
1101 GAGGTCACCT CAGGTTAACT CTGCAGCTCG TTGTCTGGAC CCTGAGCCTT  
1151 CAGCATGGCC TAATAGGCAG AGGTGGAGG GTCCTGCATA CTATTGGCGA  
1201 GGCCACC AAA GGTGCTGCTG CCCCAACCTG TCTGGCCTCC TCAACTCCCC  
1251 CACTCAGGTG CATTACACTC AGTAGGTTTG GCAAACACAG CTTCCGGTGC  
1301 TCATTTGGGA TCCTAAGGAG CAAGAGTGGG GTGAAGGGAG TGGGGAGATG  
1351 GTGTGCCGGG GAGACTGACT GCAAGCCAGT ACTTGACCGT TGCTAGAAAC  
1401 CTGTGTCAC TACAACCTGGA GCCCGGCTCC TATTACTTCA TGCCTGATGG  
1451 TCGCTGTTAT AGTCGGTCTA CAGAGGGGAA CTCCTGTCTC CCCAGGGTTG  
1501 TCATGACAGC CTTTGTGGA AGAGAGCAGG AGAACATGAC ACGTATGATG  
1551 GAGTGTGTAC ATCCCTGCCA GTGGTCCTGC TGGGGGAATC AGTGATGGGA  
1601 TAAATGTGTG CATCCCTGCA GTGGTCCTGC TGGGGGATCA GTGATGGGAT  
1651 GGGGCAGAGC CCCTATTTC TTAGAGAACT CTAACCCAAA TAAGGAACTG

FIG. 1B-2

1701 AGCCCTCTGC AGTGAGGGCT TCTGAAAACC CTGTACATAG CAAACTGTGT  
 1751 GCCCTCTTCA TCATGCAGTC CCCACCTCCT GATTCTCGGG ATGGAACCTGA  
 1801 CTTTGGTTG GAATGAAATA GACGCTCATG ATGG

### FIG. 1B-3

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1 MAAVITWALA LLSVFATVQA RKSFWEYFGQ NSQKGMMGQ QOKLAQESLK  
 51 GSLEQDLYNM NNFLEKLGPL REPGKEPPRL AQDPEGIRKQ LQOELEEVST  
 101 RLEPYMAAKH QQVGNLEGL RQQLKPYTVE LMEQVGLSVQ DLQEQLRMVG  
 151 KGTKAQLLGG VDEAMSLQD MQSRVLHHTD RYKELFHPYA ERLVTGIGHH  
 201 VQELHRSVAP HAVASPARLS RCVQTLSHKL TRKAKDLHTS IQRNLDQLRD  
 251 ELSTFIRVST DGADNRDSDL PQALSDEVRO RLQAFRHDTY LQIAAFTQAI  
 301 DQETEEIQHQ LAPPPSHSA FAPELGHSDS NKALSRLQSR LDDLWEDIAV  
 351 GLHDQGHSON NPEGHSG\*

### FIG. 2

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EPO - DG 1

27. 09. 1999

(67)

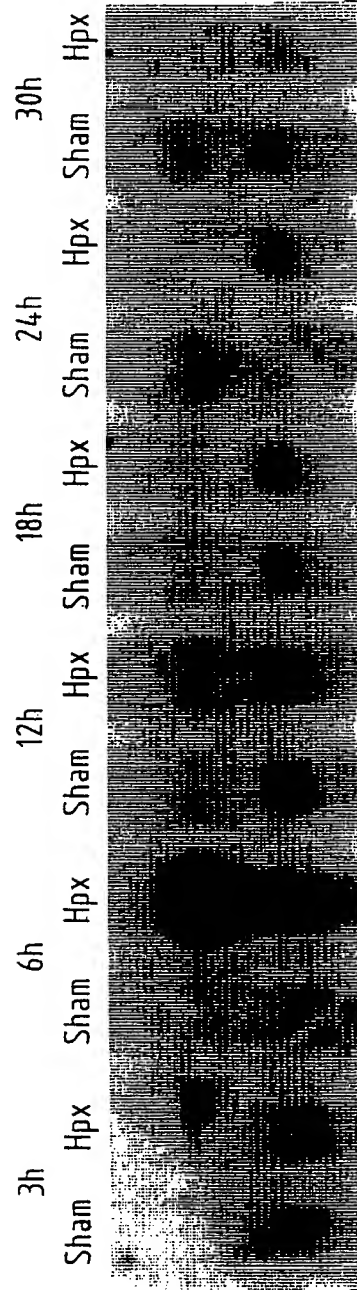


FIG. 4A

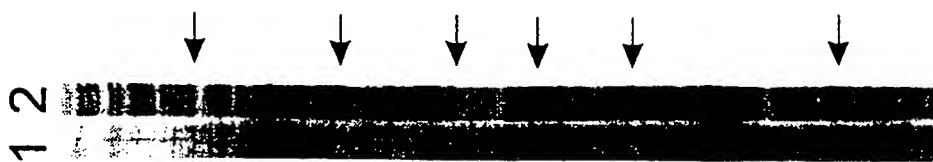
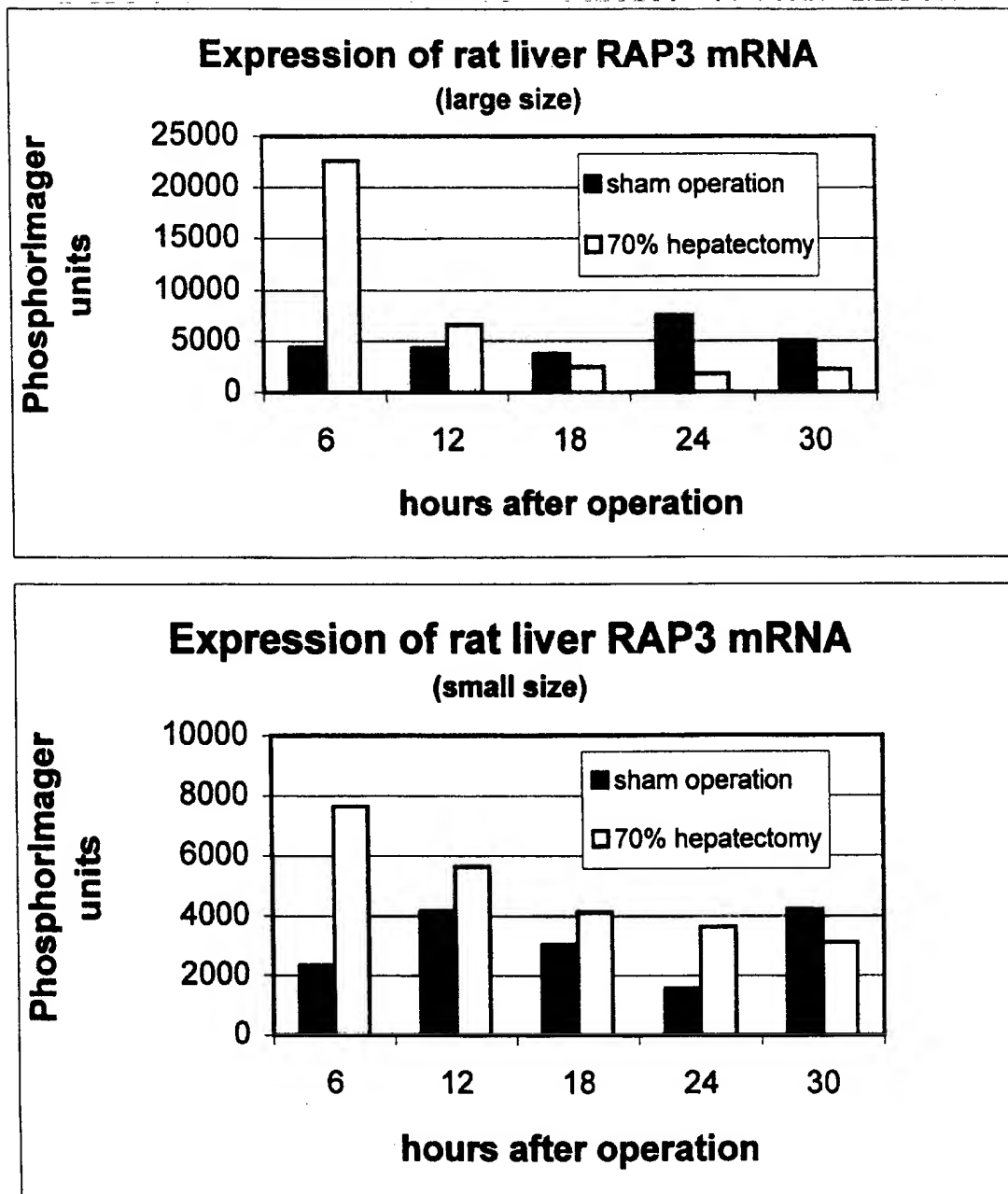


FIG. 3

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FIG. 4B



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Skeletal muscle    Spleen    Liver    Kidney    Heart    Lung    Brain



FIG. 5

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1 GAGCAGATAA TGGCAAGCAT GGCTGCCGTG CTCACCTGGG CTCGTGGCTCT  
51 TCTTTCAGCG TTTTCGGCCA CCCAGGCACG GAAAGGCTTC TGGGACTACT  
101 TCAGCCAGAC CAGCGGGGAC AAAGGCAGGG TGGAGCAGAT CCATCAGCAG  
151 AAGATGGCTC GCGAGCCCGC GACCCTGAAA GACAGCCTTG AGCAAGACCT  
201 CAACAATATG AACAACTTCC TGGAAAAGCT GAGGCCCTCTG AGTGGGAGCG  
251 AGGCTCCTCG GCTCCACACAG GACCCGGTGG GCATGCGGCG GCAGCTGCAG  
301 GAGGAGTTGG AGGAGGTGAA GGCTCGCCTC CAGCCCTACA TGGCAGAGGC  
351 GCACGAGCTG GTGGGCTGGA ATTTGGAGGG CTTGCGGCAG CAACTGAAGC  
401 CCTACACGAT GGATCTGATG GAGCAGGTGG CCCTGCCCGT GCAGGAGCTG  
451 CAGGAGCAGT TGC CGGTGGT GGGGAAGAC ACCAAGGCC AGTTGCTGG  
501 GGGCGTGGAC GAGGCTTGG CTTTGCTGCA GGGACTGCAG AGCCGCGTGG

FIG. 6A-1

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551 TGCACCACAC CGGCCGCTTC AAAGAGCTCT TCCACCCATA CGCCGAGAGC  
601 CTGGTGAGCG GCATCGGGCG CCACGTGCAG GAGCTGCACC GCAGTGTGGC  
651 TCCGCACGCC CCGGCCAGCC CCGCGCGCCT CAGTCGCTGC GTGCAGGTGC  
701 TCTCCCGGAA GCTCACGCTC AAGGCCAAGG CCTGACAGC ACGCATCCAG  
751 CAGAACCTGG ACCAGCTGCG CGAAGAGCTC AGCAGAGCCT TTGCAGGCAC  
801 TGGGACTGAG GAAGGGGCCG GCCCGGACCC CCAGATGCTC TCCGAGGAGG  
851 TGCGCCAGCG ACTTCAGGCT TTCCGCCAGG ACACCTACCT GCAGATAGCT  
901 GCCTTCACTC GCGCCATCGA CCAGGAGACT GAGGAGGTCC AGCAGCAGCT  
951 GCGGCCACCT CCACCAGGCC ACAGTGCCCTT CGCCCCAGAG TTTCAACAAA  
1001 CAGACAGTGG CAAGGTTCTG AGCAAGCTGC AGGCCCGTCT GGATGACCTG  
1051 TGGGAAGACA TCACTCACAG CCTTCATGAC CAGGGCCACA GCCATCTGGG

FIG. 6A-2

1101 GGACCCCTGA GGATCTACCT GCCCAGGCC ATTCCCAGCT CCTTGTCTGG  
1151 GGAGCCCTTG CTCTGAGCCT CTAGCATGGT TCAGTCCTTG AAAGTGGCCT  
1201 GTTGGGTGA GGTGGAAGG TCCTGTGCAG GACAGGAGG CCACCAAAGG  
1251 GGCTGCTGTC TCCTGCATAT CCAGCCTCCT GCGACTCCCC AATCTGGATG  
1301 CATTACATTC ACCAGGCTTT GC

FIG. 6A-3

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1 GAGCAGATAA TGGCAAGCAT GGCTGCCGTG CTCACCTGGG CTCTGGCTCT  
51 TCTTTCAGCG TTTTCGGCCA CCCAGGCACG GAAAGGCTTC TGGGACTACT  
101 TCAGCCAGAC CAGCGGGAC AAAGGCAGGG TGGAGCAGAT CCATCAGCAG  
151 AAGATGGCTC GCGAGCCCGC GACCCTGAAA GACAGCCTTG AGCAAGACCT  
201 CAACAATATG AACAAATTCC TGGAAAAGCT GAGGCCTCTG AGTGGGAGCG  
251 AGGTCCTCG GCTCCCACAG GACCCGGTGG GCATGCGCG GCAGCTGCAG  
301 GAGGAGTTGG AGGAGGTGAA GGCTCGCCTC CAGCCCTACA TGGCAGAGGC  
351 GCACGAGCTG GTGGGCTGGA ATTTGGAGGG CTTGCCGCAG CAACTGAAGC  
401 CCTACACGAT GGATCTGATG GAGCAGGTGG CCTGCGCGT GCAGGAGCTG  
451 CAGGAGCAGT TGC GCGTGGT GGGGAAGAC ACCAAGGCC AGTGCTGGG  
501 GGGCGTGGAC GAGGCTTGG CTTTGCTGCA GGGACTGCAG AGCCGCGTGG

FIG. 6B-1

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551 TGCACCACAC CGGCCGCTTC AAAGAGCTCT TCCACCCATA CGCCGAGAGC  
601 CTGGTGAGCG GCATCGGGCG CCACGTGCAG GAGCTGCACC GCAGTGTGGC  
651 TCCGCACGCC CCCGCCAGCC CCGCGCGCCT CAGTCGCTGC GTGCAGGTGC  
701 TCTCCCGGAA GCTCACGCTC AAGGCCAAGG CCCTGCACGC ACGCATCCAG  
751 CAGAACCTGG ACCAGCTGCG CGAAGAGCTC AGCAGAGCCT TTGCAGGCAC  
801 TGGGACTGAG GAAGGGGCGG GCCCGGACCC CCAGATGCTC TCCGAGGAGG  
851 TCGGCCAGCG ACTTCAGGCT TTCGCGCAGG ACACCTACCT GCAGATAGCT  
901 GCCTTCACTC GCGCCATCGA CCAGGAGACT GAGGAGGTCC AGCAGCAGCT  
951 GCGGCCACCT CCACCAGGCC ACAGTGCCCTT CGCCCCAGAG TTTCAACAAA  
1001 CAGACAGTGG CAAGGTTCTG AGCAAGCTGC AGGCCCGTCT GGATGACCTG  
1051 TGGGAAGACA TCACTCACAG CCTTCATGAC CAGGGCCACA GCCATCTGGG

FIG. 6B-2

1101 GGACCCCTGA GGATCTACCT GCCCAGGCC ATTCCAGCT CCTTGTCTGG  
1151 GGAGCCTTGG CTCTGAGCCT CTAGCATGGT TCAGTCCTTG AAAGTGGCCT  
1201 GTTGGGTGGA GGGTGAAGG TCCTGTGCAG GACAGGGAGG CCACCAAAGG  
1251 GGCTGCTGTC TCCTGCATAT CCAGCCTCCT GCGACTCCCC AATCTGGATG  
1301 CATTACATTC ACCAGGCTTT GCAAACCCAG CCTCCCAGTG CTCATTGGG  
1351 AATGCTCATG AGTTACTCCA TTCAAGGGTG AGGAGTAGG GAGGGAGAGG  
1401 CACCATGCAT GTGGGTGATT ATCTGCAAGC CTGTTTGCCG TGATGCTGGA  
1451 AGCCTGTGCC ACTACATCCT GGAGTTTGGC TCTAGTCACT TCTGGCTGCC  
1501 TGGTGGCCAC TGCTACAGCT GGTCCACAGA GAGGAGCACT TGTCTCCCCA  
1551 GGGCTGCCAT GGCAGCTATC AGGGAATAG AAGGGAGAAA GAGAATATCA  
1601 TGGGGAGAAC ATGTGATGGT GTGTGAATAT CCCTGCTGGC TCTGATGCTG

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FIG. 6B-3

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1651 GTGGGTACGA AAGTGTGGG CTGTGATAGG AGAGGGCAGA GCCCATGTTT  
1701 CCTGACATAG CTCTACACCT AAATAAGGA CTGAACCCCTC CCAACTGTGG  
1751 GAGCTCCTTA AACCTCTGG GGAGCATACT GTGTGCTCTC CCCATCTCCA  
1801 GCCCCTCCCT CTGGGTTCC AAGTTGAAGC CTAGACTTCT GGCTCAAATG  
1851 AAATAGATGT TTATGAT

FIG. 6B-4



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1 MAAVLTWALA LLSAFSATQA RKGFWDFYFSQ TSGDKGRVEQ IHQQKMAREP  
51 ATLKDSLEQD LNNMNKFLEK LRPLSGSEAP RLPQDPVGM RQLQEELEEV  
101 KARLQPYMAE AHELVGWNLE GLRQQLKPYT MDLMEQVALR VQELQEQLRV  
151 VGEDTKAQLL GGVDEAWALL QGLQSRVVHH TGRFKELFHP YAESLVSGIG  
201 RHVQELHRSV APHAPASPAR LSRCVQVLSR KLTLKAKALH ARIQQNLDQL  
251 REELSRFAG TGTEEGAGPD PQMLSEEV RQAFRQDTY LQIAAFTRAI  
301 DQETEEVQQQ LAPPPGHSA FAPEFQQTDS GKVL SKLAR LDDLWEDITH  
351 SLHDQGHSHL GDP\*

FIG. 7

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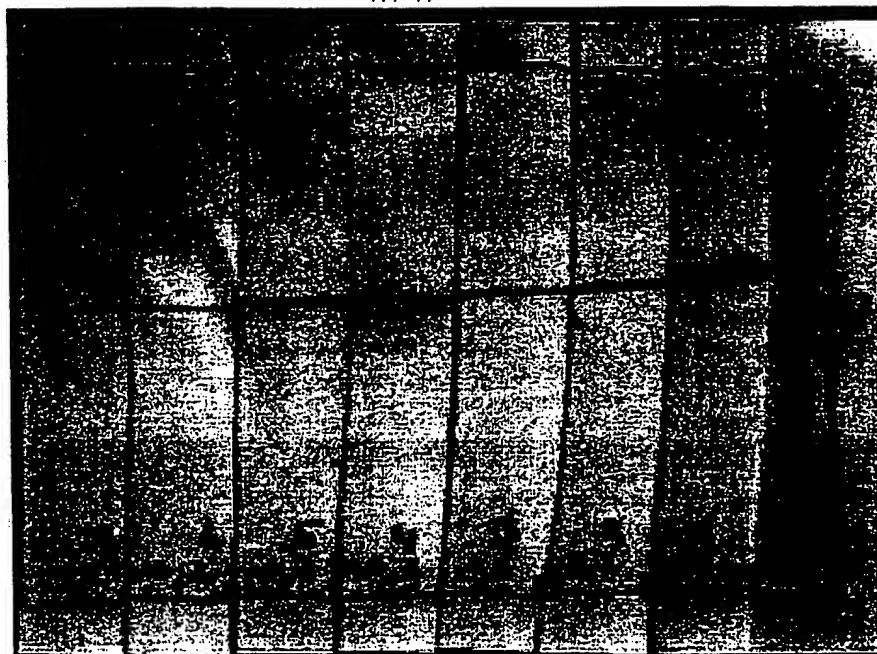


FIG. 8

3h		6h		pos.	12h	
hpx	sham	hpx	sham	contr.	hpx	sham

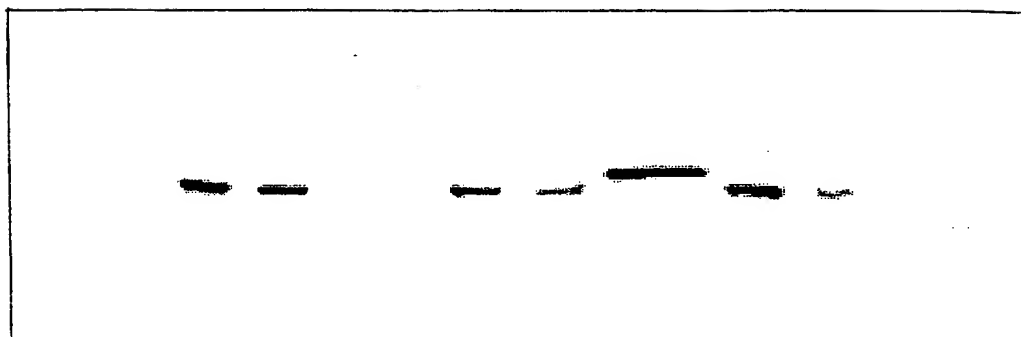


FIG. 9